Substrate Specificity of D-Galactose Oxidase

EVIDENCE FOR THE OXIDATION OF INTERNALLY LINKED GALACTOSYL RESIDUES OF HELIX POMATIA GALACTOGEN*

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Linkage analysis of the carbohydrate portion of glycoproteins and glycolipids is widespread. Sequential treatment with D-galactose oxidase and tritiated borohydride is a standard method for incorporation of radioactive marker into what has been assumed to be exclusively terminal residues of D-galactose or N-acetyl-D-galactosamine.

The data presented here establishes the ability of D-galactose oxidase to act upon a specific subterminal D-galactosyl residue, [→2]-D-Gal(1→1), as well as upon terminal nonreducing galactosyl residues. Helix pomatia galactogen, a high molecular weight galactose homopolymer, was sequentially treated with D-galactose oxidase and tritiated borohydride. The 3H-galactogen was recovered and analyzed to determine which galactosyl units carried radioactive label. After complete methylation and then acid hydrolysis of 3H-galactogen, its partially methylated galactosyl components were reduced and acetylated for identification by gas chromatography and mass spectroscopy. Radioactivity was located by collection of effluent fractions during gas chromatography. The only subterminal residue to be labeled was the 2-linked β-galactose, although another with a free oxidizable 6-carbon was present, 3-linked D-galactose, [→3]-D-Gal(1→1).

Linkage analysis of internal radioactively labeled galactosyl residues could be used to detect changes in saccharide structure during cellular events.

Sequential treatment of intact cells or isolated glycoconjugates with D-galactose oxidase and tritiated borohydride has been widely used as a means of incorporating radiolabel into cell surface glycoproteins and glycolipids (1–4). Based on substrate specificity studies performed using oligosaccharides, polysaccharides, and glycolipids (2, 5), it has been assumed that D-galactose oxidase reacts solely with terminal D-galactosyl and N-acetyl-D-galactosaminyl residues. With few exceptions (1, 2) no attempts have been made to establish whether the galactosyl residues thus labeled were present exclusively at the nonreducing termini of specific glycoconjugates.

During the course of a study on the enzymic synthesis of Helix pomatia galactogen,1 this high molecular weight galactose homopolymer was subjected to treatment with D-galactose oxidase and [3H]KBH4. Methylation analysis of the 3H-galactogen preparation revealed terminal nonreducing D-galactose to be labeled as expected (6). However, approximately one-third of the total radiolabel was present in an internally linked D-galactosyl residue substituted at the C-2 position: [→2]-D-Gal(1→1).2 Another residue, [→3]-D-Gal(1→1), with the C-6 position available for oxidation and reduction, did not become labeled. D-Galactose oxidase has also been reported by Gathmann and Aminoff (7) to act upon the subterminal β-linked galactosyl residue of the trisaccharide α-L-Fuc(1→2)-β-D-Gal(1→3)-D-GalNacol.

EXPERIMENTAL PROCEDURES

Materials—D-Galactose oxidase (EC 1.1.1.39), horseradish peroxidase (EC 1.11.1.7), and catalase (EC 1.11.1.6) were purchased from Sigma. [3H]KBH4 (1550 mCi/mmol) was a product of New England Nuclear. Aquacide 31A was obtained from Calbiochem-Behring and used according to the manufacturer's instructions. Sephadex LH-20 was purchased from Pharmacia. Whatman No. 1MM papers were products of Whatman. Plastic TLC plates precoated with silica gel were obtained from Brinkmann Instruments. Radiolabeled samples on paper or TLC plates were counted in a scintillation fluid which contained 4 g of 2,5-diphenyloxazole/liter. Radiolabeled samples in solution were counted in ACS counting scintillant from Amersham Corp.

Galactogen Preparation—Galactogen, available from a previous study, was isolated from albumen glands of adult H. pomatia as described earlier (8, 9). In this procedure albumen glands were solubilized in 5 N KOH at 100 °C, cooled, and polysaccharide precipitated with 2 volumes of absolute ethanol. After thrice resolution in water and reprecipitation with ethanol, the polysaccharide was dialyzed against distilled water overnight, recovered by a final precipitation with ethanol. The white galactogen powder was rinsed in ethanol, then in ether, and dried in a vacuum desiccator. Paper chromatography; ms, mass spectroscopy.

1E. M. Goudsmit, and D. A. Blake, manuscript in preparation.
2The abbreviations used are: Gal, galactose; Fuc, fucose; GalNacol, N-acetyl-D-galactosaminyl; TLC, thin layer chromatography; GC, gas chromatography; MS, mass spectroscopy.
graphic and enzymatic analyses of acid hydrolyses showed galactose to be the only component present (8, 9).

Galactogen Oxidation.—The ability of d-galactose oxidase to act upon galactogen was first assessed using a spectrophotometric assay (10). The reaction mixture, prepared in a 1-ml quartz cuvette, contained 34 μg (8.3 units) of galactose oxidase, 168 μg of horseradish peroxidase, 37 μg of O-tolidine, 0.1% Triton X-100, and 10 mM citrate buffer, pH 6.0, in a total volume of 950 μl. After equilibration at room temperature, 50 μl of substrate were added and the change in absorbance at 425 nm recorded by a double beam Cary spectrophotometer. The reaction, followed for 10 min at room temperature, was linear with increasing galactogen concentrations, between 0 and 14 mM, and the \( K_m \) for galactogen was calculated to be 28 mM.

Preparation of \(^3\H\)-Galactogen.—The oxidation reaction was carried out in a sterile loosely capped 5-ml Falcon test tube at room temperature. The incubation mixture contained 3.0 mg of galactose oxidase, 0.3 mg of catalase, 7.5 mg of galactogen, 0.1 M phosphate buffer, pH 7.0, and 30 μl of toluene in a 3.0-ml total volume. After incubation for 54 h an aliquot was removed, heated at 60 °C for 3 min to inactivate catalase, and then assayed spectrophotometrically for the presence of any remaining oxidizable D-galactose. None was detected.

For reduction with tritiated potassium borohydride the incubation mixture containing oxidized galactogen was brought to pH 10.0 with 1 N NaOH. [\(^3\H\)]KBH\(_4\), (10 μmol, 12.5 μCi) was added. After standing for 30 min at 41 °C, 5.0 mg of cold potassium borohydride dissolved in 0.2 ml of 0.1 N NaOH was added and the incubation continued an additional 30 min. Excess borohydride was destroyed by slowly adding 1 N H\(_2\)SO\(_4\) until the pH reached 6.0. The product, \(^3\H\)-galactogen, was purified by dialysis against distilled water overnight and then completely solubilized in 7.0 ml of dimethyl sulfoxide by sonication. Twice methylated galactogen was finally purified by column chromatography on Whatman No. 1MM paper, 1% sodium tetraborate, pH 9.2, 4000 V for 30 min. All the hydrolyzed tritiated material in the Hakomori method mixture containing oxidized galactogen was brought to pH 10.0 with 1 N NaOH. [\(^3\H\)]KBH\(_4\), (10 μmol, 12.5 μCi) was added. After standing for 30 min at 41 °C, 5.0 mg of cold potassium borohydride dissolved in 0.2 ml of 0.1 N NaOH was added and the incubation continued an additional 30 min. Excess borohydride was destroyed by slowly adding 1 N H\(_2\)SO\(_4\) until the pH reached 6.0. The product, \(^3\H\)-galactogen, was purified by dialysis against distilled water overnight and then completely solubilized in 7.0 ml of dimethyl sulfoxide by sonication. Twice methylated galactogen was finally purified by column chromatography on Whatman No. 1MM paper, 1% sodium tetraborate, pH 9.2, 4000 V for 30 min. All the hydrolyzed tritiated material was analyzed by high voltage electrophoresis (Gibson model D on Whatman No. 3MM paper, 1% sodium tetaborate, pH 9.2, 4000 V for 30 min) and by descending paper chromatography on Whatman No. 1MM paper in butanol/pyridine/water (6:4:3). All the hydrolyzed tritiated material co-chromatographed and co-electrophoresed with an internal \(^1^4\)C galactose standard.

Methylation of \(^3\H\)-Galactogen.—Tritiated galactogen (2.5 × 10\(^7\) cpm) was mixed with 4.5 mg of carrier galactogen for methylation by the Hakomori method (12). Unlabeled galactogen (5.0 mg) was carried through identical procedures. In preparation for methylation, samples were completely dried by evaporation over phosphorus pentoxide and then completely solubilized in 7.0 ml of dimethyl sulfoxide by sonication for 3 h at 60 °C. Methylsulfinylcarbanion (2.5 ml) was added with stirring, followed after 22 h by the addition of 2.0 ml of methyl iodide. The methylation reaction continued for 12 h. The product was purified by dialysis, lyophilized, and the Hakomori methylation repeated. Twice methylated galactogen was finally purified by column chromatography on Sephadex LH-20 prepared in chloroform:methanol (1:1) (13, 14). An infrared spectrum of 0.5 mg of methylated galactogen in 50.0 mg of KBr showed the reaction to be complete. Recovery of methylated \(^3\H\)-galactogen was 60%, based on radioactivity.

RESULTS

Analyses of Methylated \(^3\H\)-Galactogen

tlc.—Methylated samples of galactogen were hydrolyzed at 100 °C for 5 h in 90% formic acid followed by 0.25 N sulfuric acid for 14 h, then neutralized with barium carbonate. tlc of partially methylated sugars obtained was performed using either solvent a (acetone, 5 N NH\(_4\)OH (100:1.8, v/v) or solvent b (acetone, benzene, water, 1 N NH\(_4\)OH (200:50:3.1.5, v/v). Carbohydrates were visualized after spraying with ethanol; formic acid hydrolysis.

Fig. 1. Thin layer chromatography of partially methylated galactose species obtained from methylated \(^3\H\)-galactogen. Top, after tlc in solvent a, sugars were visualized with ethanol:H\(_2\)SO\(_4\) spray. Migration positions of standards are indicated by arrows. Standards were available from a previous study (15). Bottom, trimethylated compounds were located in a parallel lane of the same chromatogram by cutting the plastic-backed tlc sheet into 15-cm segments and counting. The slower moving radioactive material was eluted from the sheet and subjected to structural characterization (see Fig. 3).

indicated two radiolabeled areas whose migration rates corresponded to 2,3,4,6-tetra-O-methyl- and 2,3,4,tri-O-methylgalactose (Fig. 1). Since we had assumed the tritium would have been confined to the tetra-O-methylgalactose (6), it seemed important to discover the identity of the second tritiated component whose radioactivity comprised about 50% of that in the tetramethylgalactose.

gc-ms—For structural analysis of the unknown tritiated component, methylated galactogens were hydrolyzed and then reduced and acetylated by the method of Stellner et al. (13). Gas chromatography was performed with a Hewlett-Packard 5840A gas chromatograph equipped with flame ionization detectors, using a 6-foot glass column (2 mm, inner diameter) packed with 3% OV-210 on Supelcoport (80–100 mesh) and temperature programmed from 150 to 230 °C at 3/°C/min. Six partially methylated alditol acetate derivatives were found (Fig. 2); 41% of 1,5-di-O-Ac-2,3,4,6-tetra-O-Me; B, 1,3,5-tri-O-Ac-2,4,6-tri-O-Me; C, 1,2,4-tri-O-Ac-3,4,6-tri-O-Me; D, 1,5,6-tri-O-Ac-2,3,4,6-di-O-Me; E, 1,3,5,6-tetra-O-Ac-2,3,4-di-Me; F, 1,2,5,6-tetra-O-Ac-3,4,6-di-O-Me. Other peaks were noncarbohydrate contaminants. See under "Experimental Procedures."
D-Galactose Oxidase Acts on Internally Linked Galactose

Identification of the Unknown Tritiated Component As 3,4,6-Tri-O-methylgalactose

2,3,4- and 3,4,6-tri-O-methylgalactose are not resolved by the tlc systems used here (18). Since the latter is one of the partially methylated galactoses from H. pomatia galactogen and would have a free oxidizable carbon at the 6-position it was hypothesized to be the unknown tritiated component.

Methylated 3H-galactogen was hydrolyzed with formic acid:H2SO4 as described above and a 200-μg (1 x 10^6 cpm) aliquot was chromatographed by tlc in solvent a. The area containing the unknown tritiated component was cut from the plastic-backed sheet, and the silica gel was scraped into a 12-ml conical Pyrex test tube. Methylated compounds were eluted with chloroform:methanol (3:1, v/v). After being dried, the compounds were reduced with NaBH4 and acetylated (13).

Analysis by gc-ms revealed that the tlc area eluted had contained 2,3,4-tri-O-methylgalactose and 3,4,6-tri-O-methylgalactose as the sole constituents (Fig. 3A). In order to determine which molecule carried the tritium, radioactivity of gc effluent was monitored as follows: the flame ionization detector was shut off and Teflon tubing inserted through the detector outlet so that the end tightly covered the jet tip. The other end of the tubing was placed in a scintillation vial containing 3 ml of chilled chloroform. Sample was injected into the gas chromatograph and volatilized effluent collected at timed intervals based on retention times of the trimethylgalactitol acetates previously calibrated with a stop watch. A new set of vials and tubing was used for each timed interval. Condensate collecting inside the tubing was washed out with 2.0 ml of chloroform into the appropriate vial. After evaporation of chloroform, 1.0 ml of scintillation fluid was added for counting. All of the radioactive material applied to the column was recovered, and it all appeared as a single peak with a retention time identical to that of alditol acetate derivative of 3,4,6-tri-O-methylgalactose (Fig. 3).

DISCUSSION

While the exact structural pattern of galactogen remains uncertain, detailed analyses by several laboratories indicate a highly branched structure of β-D-(1→3) and β-D-(1→6) linkages (16, 19-21). Galactogen of the land snail H. pomatia is unusual in that approximately 14% of total galactose is the L-isomer, which occupies a nonreducing terminal position and

Identification of the Unknown 3H-Labeled Component of Methylated 3H-Galactogen

A, gas chromatogram of partially methylated alditol acetate derivatives of the methylated compounds eluted from tlc (see Fig. 2). Inset, radioactivity (cpm) recovered in each fraction is shown by dashed vertical lines. B, mass spectrum of peak I, identified as 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylgalactitol. C, mass spectrum of peak II, identified as 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylgalactitol. See under “Experimental Procedures” for details.
is linked α-(1→2) to subterminal D-galactose (19, 21).

The experimental data presented herein on the preparation and analysis of 3H-labeled H. pomatia galactogen indicate that a specific internally linked galactosyl residue, nonreducing terminal galactose, became tritiated by the galactose oxidase procedure. The internally linked residue was isolated and unequivocally identified in its partially permethylated form as 3,4,6-tri-O-methylgalactose, i.e. [→2]-D-Gal-(1→). Analyses by others (16, 19, 21) have established that this galactose is penultimate and linked to nonreducing terminal L-galactose. The 2-linked D-galactosyl residue was the only subterminal residue to become labeled, although another with a free oxidizable 6-carbon was present [→3]-D-Gal(1→).

Thus it appears that a (1→2)-linked penultimate D-galactosyl residue is within the catalytic capabilities of D-galactose oxidase. This subterminal residue may be substituted at the C-2 position with L-galactose or with L-fucose (6-deoxy-L-galactose) as shown by Gathmann and Aminoff (7).

D-Galactose oxidase is a powerful tool for the incorporation of radiolabel into cell surface glycoproteins and glycolipids. The data presented herein argue for caution in the interpretation of changes in labeling patterns as a means of determining oligosaccharide structure, if based solely on susceptibility to D-galactose oxidase. Identification of labeled internally linked components should be carried out and could yield important data on changes in saccharide structure during events such as the cell cycle or transformation. We have found that the identification of labeled internal galactosyl residues has allowed us to detect complex changes in the structure of H. pomatia galactogen during its enzymic synthesis.

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